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DEVELOPMENT OF A CDNA BASED RAPID SCREENING METHOD FOR ASCOCHYTA BLIGHT RESISTANCE IN LENTIL (LENS CULINARIS L.)

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Development of a cDNA Baser Rapid Screening Method for Ascochyta Blight Resistance in Lentil

Final Report

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Abstract

The ultimate goal of this project was to develop a fast, reliable and low cost cDNA based molecular marker system for screening lentil breeding lines for resistance to *Ascochyta* blight so that resistant lines can be reliably identified and introduced into the lentil breeding program.

We extracted pathogen induced messenger RNA samples from a highly susceptible variety (CDC Richlea) and a highly resistant breeding line (1156-2-17A) for comparison using a molecular biological tool called differential display. 152 DNA bands representing genes expressed or suppressed 24 hours after inoculation with *Ascochyta lentis* were identified as potentially useful and excised from gels for re-amplification and cloning. These bands were categorized as those which are induced, upregulated, suppressed or downregulated in the resistant line 1156-2-17A. Two of the bands represent genes that were constitutively expressed in the susceptible variety Richlea and three bands represent genes constitutively expressed by the resistant line. Seventy gel bands have been successfully re-amplified and cloned into cloning vectors. All cloned DNA fragments were sequenced, including multiple clones sequenced from some bands.

We planted the recombinant inbred lines derived from a cross 964a-46X CDC Robin. Thirty seven RILs containing different combinations were screened for ascochyta blight resistance along with the parents of the RIL population, CDC Richlea and line 1156-2-17A. RNA extracted from the tissues collected from 14 of these lines can be used in future for validating the results. The primers developed from the differential display study can be used to test whether they are useful as markers regardless of the genetic sources of resistance. Alternatively they may be useful in distinguishing between the two sources of resistance to ascochyta blight- namely *ral*1 and *Ab*R1.

Executive Summary

- Successful differential display experiments were conducted to compare expressed genes in control and pathogen (*Ascochyta lentis* isolate 80982) inoculated tissue of the susceptible lentil variety Richlea and the resistant breeding line 1156-2-17A
- 152 DNA bands of cDNA fragments representing genes differentially expressed in susceptible and resistant lines of lentil during pathogen infection were isolated.
- Seventy gel bands were cloned, sequenced, and then used for similarity (BLAST) searches against available DNA and protein sequences in public databases.
- Thirty-five of the sequenced gene fragments showed similarity to known genes; 20 sequences had no similar sequences in databases; 15 sequences were either too short or of poor quality.

- Several differential display gel bands were similar in sequence to pathogen induced genes in soybean, medicago and other plants. These sequences are good candidates for screening susceptible and resistant lines for differential expression.

- Primers based on these sequences will be useful in detecting resistant lines using RT PCR experiments.

- A population of RILs carrying the *ral1* and *Ab*R1 sources of resistance to ascochyta blight resistance were planted, and then screened for resistance to *Ascochyta lentis* isolate 80982.

- Tissue was collected from 14 RIL lines 24 hrs after inoculation with *Ascochyta lentis* isolate 80982 for future testing of how useful the technique will be for differentiating among lines carrying various combinations of the two resistance genes.

Technical Report

Objective:

The objective of this project was to use the molecular biological technique known as differential display to identify and sequence the genes that are uniquely expressed by the ascochyta blight resistant lentil line 1156-217A when compared to the highly susceptible lentil variety Richlea. The sequences of this set of 'resistance related genes' will be used to design an RT PCR based molecular screening system to identify individual disease resistant plants in breeding populations at the seedling stage.

Project Activities and Results:

Standardization of plant growth and inoculation conditions:

Fourteen to 18 day old plants of the ascochyta blight resistant lentil line 1156-2-17A and the susceptible variety Richlea were grown and inoculated with spores from cultures of *A. lentis* (isolate No. 80982). Uninoculated controls were sprayed with sterile distilled water. The inoculated plants were scored for disease symptoms (lesions) on the 14th day after inoculation. Based on the number of lesions per plant induced over a range of spore concentrations between 500,000 spores/mL to 6250 spores/mL, a clear differential reaction to the pathogen was observed in susceptible and resistant lines. The susceptible variety Richlea showed severe symptoms of disease development regardless of the concentration of spores used, while the resistant line 1156-2-17A showed increasingly severe symptoms as the spore concentration was increased. No tissue damage could be observed in either of the lines up to 72 hours after inoculation with any of these concentrations.

Collection and storage of lentil tissue samples:

Based on the results from our standardization experiments, we inoculated four different sets of 1156-2-17A and Richlea plants with different concentrations of *A. lentils* spores as described in the previous section. Ten to 15 gram samples of tissue were harvested from each set of plants 10, 24 and 72 hours after inoculation. The tissue were frozen immediately in liquid nitrogen and stored for future use at –70 °C. One control set was treated with a spray of sterile distilled water under the same conditions as the inoculated samples. A few randomly chosen plants from each treatment were left to grow for 14 days after inoculation and disease symptoms were confirmed on the inoculated samples to ensure that inoculation was successful.

Isolation of high quality RNA from control and infected lentil plants

RNA was isolated from control and ascochyta-inoculated lentil tissue using the RNAeasy mini-kit manufactured by Qaiagen Inc., Mississauga, Ontario. The RNA was tested for quality using formaldehyde agarose gels and found to be of good quality. However each differential display experiment would require about 30 µg RNA. Based on the basic methodology for extracting high quality RNA standardized earlier, larger RNA preparations were made from all the available samples using the Qaiagen midi RNA extraction kits. The total amount of RNA extracted from these tissue ranges from 50 to 100 micrograms. The RNA from these preparations was tested by running formaldehyde agarose gels and found to be of good quality.

Differential Display Experiments:

In order to generate differential display gels, we needed to reverse transcribe and amplify the expressed genes from total RNA preparations. The reverse transcription step used a polyT primer with a terminal 3' A, G or C nucleotide (named HT11A or HT11C or HT11 G). Several PCR reactions were then carried out using the original reverse transcription reaction mixture as a template with the original HT11N primer plus a series of random primers (HAP1 to HAPn) on the reverse. The PCR products generated from the RNA extracted from different tissues were run on adjacent lanes a 6% polyacrylamide gel. Differential bands were identified, recorded and cut out from the gel to be cloned. The cDNA fragment extracted from the gel was re-amplified, cloned and sequenced.

Since the differential display reactions are quite sensitive to the quality and concentrations of different components, standardization of the reactions was initially difficult to achieve in our laboratory. The first two attempts to carry out the reactions were unsuccessful. That compelled us to re-check the quality of all our reagents and RNA preparations with control samples obtained from other laboratories working with plant RNA. This caused some delay in our ability to conduct differential display reactions.

The detection of DNA on differential display gels is traditionally done with the aid of radioactively labeled nucleotides incorporated at the PCR stage. However, to avoid the hazard and expense of working with radioactivity, we initially opted for the fluorescence labeled primer based detection system newly developed by the supplier of the kit (GenHunter Corporation). We used the BIORAD Molecular Imager FX (kindly made available by Dr. Isabel Parkin, Agriculture and Agri-Food Canada, Saskatoon Research

Station) to visualize the gels. However, the sensitivity of this instrument was not sufficient to visualize differential display bands on large polyacrylamide gels.

Based on extensive consultations with the supplier of the differential display kit and other laboratories with previous experience in differential display, we successfully standardized the silver staining protocol for this purpose and DNA bands were extracted from silver stained gels which we successfully re-amplified and cloned.

Reverse transcription using polyT primers:

Four sets of reverse transcription reactions were carried out using RNA extracted from inoculated and control lentil plants.
RT reaction 1: In the first reaction RNA extracted from tissues of 1156-2-17A (resistant) before and after inoculation from the control plants were reverse transcribed using the primer HT11A. Control RNA from two other sources were also included.
RT reaction 2: In the second reaction, RNA extract from Richlea and 1156-2-17A tissue, harvested 24 hours after inoculation were used along with the controls. The primers used for this reaction were HT11A, HT11C and HT11G.
RT reactions 3 and 4: In the third and fourth reactions, RNA extracted from Richlea and 1156-2-17A tissue, harvested 24 hours after inoculation were used along with the controls. The primers used for this reaction were HT11A, HT11C and HT11G. These reactions were conducted to generate a sufficient stock of reverse transcription products for future PCR reactions.

PCR using HT11N and random primers:

A total of 320 PCR reactions were conducted in eight different PCR experiments using the product of reverse transcription reactions 1 to 4 as template.
PCR1: This reaction was conducted to check if the differential display reactions were working. The fluorescent Rhodamine labeled RHT11A primer and the reverse random primer HAP9 were used to amplify the reverse transcribed RNA samples from reverse transcription reaction 1. One part of each reaction was run out on a 0.8% agarose gel with ethidium bromide. The PCR product generated by the reaction could be visualized using a UV Trans illuminator, confirming that the differential display reactions were working. One part was run on a 0.8% agarose gel without agarose gel and visualized in a Molecular Imager to check if the fluorescent label was detectable using an Imager. The concentrated bands of PCR products were visible as fluorescent bands in the imager. The remaining part of the reactions were run on a AFLP type 6% polyacrylamide denaturing gel as required for differential display. This gel was scanned for fluorescent bands using the BIORAD Molecular Imager FX. No DNA bands were visible on the larger gel leading us to conclude that the fluorescent sensitivity of the instrument was insufficient to detect differential display bands.

PCR2: This reaction was also used the reverse transcription product from RT reaction 1. However, only the RT products from 1156-2-17A control and 1156-2-17A inoculated (24 h) were used in this PCR. The HT11A (unlabelled) primer and the reverse random primers HAP1 to HAP8 were used to amplify the reverse transcribed RNA for differential display. The PCR products from these reactions (16 in all) were run out on a

6% polyacrylamide gel and individual PCR products separated by molecular weight. The gel was developed using standard silver staining protocols used for AFLP gels and individual bands visualized (See attached Fig.1).

PCR3: This reaction used the reverse transcription products from RT reaction 2. The RNA templates used were extracted from Richlea and 1156-2-17A plants 24 hours after inoculation. Control RNA was used for comparison. The HT11A, HT11C and HT11G (unlabelled) primers and the reverse random primers HAP1 and HAP2 were used to amplify the reverse transcribed RNA for differential display. The PCR products from these reactions (24 in all) were run out on a 6% polyacrylamide gel and individual PCR products separated by molecular weight. The gel was developed using standard silver staining protocols used for AFLP gels and individual bands visualized.

PCR4 to 8: This reaction used the reverse transcription products from RT reaction 3 to4. The RNA templates used were extracted from Richlea and 1156-2-17A plants12 and 24 hours after inoculation. Control RNA was used for comparison. The HT11A, HT11C and HT11G (unlabelled) primers and the reverse random primers HAP1 and HAP2 were used to amplify the reverse transcribed RNA for differential display. The PCR products from these reactions (24 in all) were run out on a 6% polyacrylamide gel and individual PCR products separated by molecular weight. The gel was developed using standard silver staining protocols used for AFLP gels and individual bands visualized.

Detection of differentially expressed genes:

DNA bands on differential display gels representing genes whose activity was affected by inoculation with Ascochyta were classified and assigned the following categories:

1) **Ir** – Induced in the resistant line (1156-2-17A) only
2) **Ur** – Upregulated in the resistant line (1156-2-17A) only
3) **Sr** - Suppressed in the resistant line (1156-2-17A) only
4) **D** – Downregulated in the resistant line (1156-2-17A) only
5) **Is** – Induced in the susceptible (Richlea) line only
6) **Us** – Upregulated in the susceptible (Richlea) line only
7) **Ss** - Suppressed in the susceptible (Richlea) line only
8) **Ds**– Downregulated in the susceptible (Richlea) line only
9) **Cs** - Constitutively expressed in susceptible line (Richlea)
10) **Cr** - Constitutively expressed in resistant line (1156-2-17A)

A total of 152 bands were identified from two different differential display gels using all three polyT (HT11N) primers and a subset of the random (HAPn) primers (Table 1). DNA from all 152 bands was extracted. Seventy bands were PCR re-amplified and successfully cloned and sequenced. The sequences have been used to search the NCBI DNA and protein databases for similarity to known gene sequences in the public domain. The sequences from this project have identified several lentil genes with similarity to known genes in the model legume *M. truncatula*, other legumes and *Arabidopsis* (Table 2). Samples of sequences are shown in the Appendix to this report. We will make all useful sequence data available in public databases.

Table 1: List of differential display bands identified from two different

differential display (DD) gels

S.No	band no.	Gel No.	Lane No	Mol.Wt(bp)	band type	DD Primers	
						Anchor primer	Arbit. Primer
1	1	3	7	108	Dr	HT11A	HAP1
2	2	3	7	965	Sr	HT11A	HAP1
3	3	3	15	689	Dr	HT11A	HAP1
4	4	3	15	555	Ur	HT11A	HAP1
5	5	3	15	550	Ir	HT11A	HAP1
6	6	3	15	544	Ur	HT11A	HAP1
7	7	3	15	544	Ur	HT11A	HAP1
8	8	3	8	853	Dr	HT11A	HAP2
9	9	3	8	786	Dr	HT11A	HAP2
10	10	3	8	740	Dr	HT11A	HAP2
11	11	3	8	710	Dr	HT11A	HAP2
12	12	3	8	517	Dr	HT11A	HAP2
13	13	3	16	985	Ur	HT11A	HAP2
14	14	3	16	803	Ir	HT11A	HAP2
15	15	3	16	740	Dr	HT11A	HAP2
16	16	3	16	703	Sr	HT11A	HAP2
17	17	3	16	561	Ir	HT11A	HAP2
18	18	3	16	439	Ir	HT11A	HAP2
19	19	3	16	272	Dr	HT11A	HAP2
20	20	3	9	210	Dr	HT11A	HAP3
21	21	3	17	1000	Ir	HT11A	HAP3
22	22	3	17	975	Ur	HT11A	HAP3
23	23	3	17	955	Ir	HT11A	HAP3
24	24	3	17	803	Ur	HT11A	HAP3
25	25	3	17	786	Ur	HT11A	HAP3
26	26	3	17	710	Ur	HT11A	HAP3
27	27	3	17	591	Ur	HT11A	HAP3
28	28	3	10	579	Dr	HT11A	HAP4
29	29	3	10	317	Sr	HT11A	HAP4
30	30	3	10	307	Sr	HT11A	HAP4
31	31	3	18	803	Dr	HT11A	HAP4
32	32	3	18	725	Ur	HT11A	HAP4
33	33	3	18	696	Ur	HT11A	HAP4
34	34	3	18	597	Ur	HT11A	HAP4
35	35	3	11	1125	Dr	HT11A	HAP5
36	36	3	11	1047	Sr	HT11A	HAP5
37	37	3	11	871	Sr	HT11A	HAP5
38	38	3	11	811	Sr	HT11A	HAP5
39	39	3	11	710	Sr	HT11A	HAP5
40	40	3	11	591	Dr	HT11A	HAP5
41	41	3	11	365	Sr	HT11A	HAP5

differential display (DD) gels

S.No	band no.	Gel No.	Lane No	Mol:Wt(bp)	band type	DD Primers	
						Anchor primer	Arbit. Primer
1	**1**	3	7	108	Dr	HT11A	HAP1
2	**2**	3	7	965	Sr	HT11A	HAP1
3	**3**	3	15	689	Dr	HT11A	HAP1
4	**4**	3	15	555	Ur	HT11A	HAP1
5	**5**	3	15	550	Ir	HT11A	HAP1
6	**6**	3	15	544	Ur	HT11A	HAP1
7	**7**	3	15	544	Ur	HT11A	HAP1
8	**8**	3	8	853	Dr	HT11A	HAP2
9	**9**	3	8	786	Dr	HT11A	HAP2
10	**10**	3	8	740	Dr	HT11A	HAP2
11	**11**	3	8	710	Dr	HT11A	HAP2
12	**12**	3	8	517	Dr	HT11A	HAP2
13	**13**	3	16	985	Ur	HT11A	HAP2
14	**14**	3	16	803	Ir	HT11A	HAP2
15	**15**	3	16	740	Dr	HT11A	HAP2
16	**16**	3	16	703	Sr	HT11A	HAP2
17	**17**	3	16	561	Ir	HT11A	HAP2
18	**18**	3	16	439	Ir	HT11A	HAP2
19	**19**	3	16	272	Dr	HT11A	HAP2
20	**20**	3	9	210	Dr	HT11A	HAP3
21	**21**	3	17	1000	Ir	HT11A	HAP3
22	**22**	3	17	975	Ur	HT11A	HAP3
23	**23**	3	17	955	Ir	HT11A	HAP3
24	**24**	3	17	803	Ur	HT11A	HAP3
25	**25**	3	17	786	Ur	HT11A	HAP3
26	**26**	3	17	710	Ur	HT11A	HAP3
27	**27**	3	17	591	Ur	HT11A	HAP3
28	**28**	3	10	579	Dr	HT11A	HAP4
29	**29**	3	10	317	Sr	HT11A	HAP4
30	**30**	3	10	307	Sr	HT11A	HAP4
31	**31**	3	18	803	Dr	HT11A	HAP4
32	**32**	3	18	725	Ur	HT11A	HAP4
33	**33**	3	18	696	Ur	HT11A	HAP4
34	**34**	3	18	597	Ur	HT11A	HAP4
35	**35**	3	11	1125	Dr	HT11A	HAP5
36	**36**	3	11	1047	Sr	HT11A	HAP5
37	**37**	3	11	871	Sr	HT11A	HAP5
38	**38**	3	11	811	Sr	HT11A	HAP5
39	**39**	3	11	710	Sr	HT11A	HAP5
40	**40**	3	11	591	Dr	HT11A	HAP5
41	**41**	3	11	365	Sr	HT11A	HAP5

S.No	band no.	Gel No.	Lane No	Mol. Wt(bp)	band type	DD Primers	
						Anchor primer	Arbit. Primer
42	**42**	3	11	233	Dr	HT11A	HAP5
43	**43**	3	23	907	Ds	HT11A	HAP1
44	**44**	3	23	384	CS	HT11A	HAP1
45	**45**	3	30	457	Ir	HT11C	HAP1
46	**46**	3	40	795	Is	HT11C	HAP2
47	**47**	3	40	512	Is	HT11C	HAP2
48	**48**	3	23,24	384	CS	HT11A	HAP1
49	**49**	3	27,28	641	CS	HT11C	HAP1
50	**50**	3	27,28	641	CS	HT11C	HAP1
51	**51**	3	29,30	634	CR	HT11C	HAP1
52	**52**	2	7	380	Ir	HT11A	HAP1
53	**53**	2	7	360	Ir	HT11A	HAP1
54	**54**	2	7	220	Ur	HT11A	HAP1
55	**55**	2	8	530	Sr	HT11A	HAP2
56	**56**	2	8	500	Dr	HT11A	HAP2
57	**57**	2	8	475	Dr	HT11A	HAP2
58	**58**	2	8	400	Sr	HT11A	HAP2
59	**59**	2	8	390	Sr	HT11A	HAP2
60	**60**	2	8	300	Sr	HT11A	HAP2
61	**61**	2	8	290	Sr	HT11A	HAP2
62	**62**	2	8	250	Sr	HT11A	HAP2
63	**63**	2	8	245	Sr	HT11A	HAP2
64	**64**	2	9	370	Ir	HT11A	HAP2
65	**65**	2	9	220	Ir	HT11A	HAP2
66	**66**	2	10	245	Sr	HT11A	HAP3
67	**67**	2	10	240	Sr	HT11A	HAP3
68	**68**	2	11	510	Ir	HT11A	HAP3
69	**69**	2	12	400	Sr	HT11A	HAP4
70	**70**	2	12	390	Sr	HT11A	HAP4
71	**71**	2	12	290	Sr	HT11A	HAP4
72	**72**	2	12	285	Sr	HT11A	HAP4
73	**73**	2	12	280	Sr	HT11A	HAP4
74	**74**	2	13	310	Ir	HT11A	HAP4
75	**75**	2	14	800	Sr	HT11A	HAP5
76	**76**	2	14	600	Sr	HT11A	HAP5
77	**77**	2	14	290	Sr	HT11A	HAP5
78	**78**	2	14	295	Sr	HT11A	HAP5
79	**79**	2	14	240	Sr	HT11A	HAP5
80	**80**	2	14	245	Sr	HT11A	HAP5
81	**81**	2	14	248	Sr	HT11A	HAP5
82	**82**	2	15	450	Ir	HT11A	HAP5
83	**83**	2	18	350	Sr	HT11A	HAP7

S.No	band no.	Gel No.	Lane No	Mol. Wt(bp)	band type	DD Primers	
						Anchor primer	Arbit. Primer
84	**84**	2	19	750	Ir	HT11A	HAP7
85	**85**	2	19	330	Ir	HT11A	HAP7
86	**86**	2	19	335	Ir	HT11A	HAP7
87	**87**	2	19	332	Ir	HT11A	HAP7
88	**88**	2	19	310	Ur	HT11A	HAP7
89	**89**	2	19	275	Ir	HT11A	HAP7
90	**90**	2	19	248	Ir	HT11A	HAP7
91	**91**	2	19	255	Ir	HT11A	HAP7
92	**92**	2	19	210	Ir	HT11A	HAP7
93	**100**	4	2	434	Sr	HT11A	HAP1
94	**101**	4	2	447	Sr	HT11A	HAP1
95	**102**	4	2	473	Sr	HT11A	HAP1
96	**103**	4	3	476	Ir	HT11A	HAP1
97	**104**	4	4	424	Sr	HT11A	HAP2
98	**105**	4	4	410	Sr	HT11A	HAP2
99	**106**	4	5	474	Ir	HT11A	HAP2
100	**107**	4	5	399	Ur	HT11A	HAP2
101	**108**	4	5	303	Ur	HT11A	HAP2
102	**109**	4	5	366	Ur	HT11A	HAP2
103	**110**	4	7	434	Ir	HT11A	HAP3
104	**111**	4	9	402	Ur	HT11A	HAP4
105	**112**	4	9	366	Ur	HT11A	HAP4
106	**113**	4	9	460	Ur	HT11A	HAP4
107	**114**	4	10	489	Sr	HT11A	HAP5
108	**115**	4	10	476	Sr	HT11A	HAP5
109	**116**	4	10	396	Ur	HT11A	HAP5
110	**117**	4	11	494	Dr	HT11A	HAP5
111	**118**	4	12	331	Sr	HT11A	HAP6
112	**119**	4	15	457	Ir	HT11A	HAP7
113	**120**	4	15	408	Ir	HT11A	HAP7
114	**121**	4	15	322	Ir	HT11A	HAP7
115	**122**	4	15	392	Ir	HT11A	HAP7
116	**123**	4	18	493	Cr Sr	HT11A	HAP1
117	**124**	4	25	460	Cr Ur	HT11C	HAP1
118	**125**	5	3	405	Cs, Ss	HT11A	HAP1
119	**126**	5	6	397	Cr, Ir	HT11A	HAP1
120	**127**	5	3	81	Cr		
121	**128**	5	7	241	Cs Ss	HT11C	HAP1
122	**129**	5	10	229	Cr	HT11C	HAP1
123	**130**	5	9	135	Cr	HT11C	HAP1
124	**131**	5	15	183	Cs	HT11A	HAP2
125	**132**	5	23	311	Ir	HT11C	HAP2

S.No	band no.	Gel No.	Lane No	Mol Wt(bp)	band type	DD Primers	
						Anchor primer	Arbit. Primer
126	**133**	5	22	114	Sr	HT11C	HAP2
127	**134**	5	9	198	Sr	HT11C	HAP1
128	**135**	5	13	194		HT11G	HAP1
129	**136**	5	11	474	Ss	HT11G	HAP1
130	**137**	5	22	239	Sr	HT11C	HAP2
131	**138**	5	23	70	Ir	HT11C	HAP2
132	**151**	6	3	559	Ss	HT11A	HAP1
133	**152**	6	4	421	Is	HT11A	HAP1
134	**153**	6	4	297	Is	HT11A	HAP1
135	**154**	6	22	297	Cs	HT11A	HAP1
136	**155**	6	22	274	Cs	HT11A	HAP1
137	**156**	6	22	222	Cs	HT11A	HAP1
138	**157**	6	22	220	Cs	HT11A	HAP1
139	**158**	6	24	462	cr	HT11A	HAP1
140	**159**	6	20	331	Ir	HT11A	HAP1
141	**160**	6	19	336	Cr	HT11A	HAP1
142	**161**	6	15	519	Cr	HT11A	HAP1
143	**162**	6	14	315	Is	HT11A	HAP1
144	**163**	6	13	214	Ss	HT11A	HAP1
145	**164**	6	9	290	Sr	HT11A	HAP1
146	**165**	6	9	287	Sr	HT11A	HAP1
147	**166**	6	8	317	Sr	HT11A	HAP1
148	**167**	6	10	403	Ir	HT11A	HAP1
149	**168**	6	8	399	Ir	HT11A	HAP1
150	**169**	6	10	207	Ir	HT11A	HAP1
151	**170**	6	10	205	Ir	HT11A	HAP1
152	**171**	6	15	446	Cr	HT11A	HAP1

Validation of differential display results:

In consultation with Dr. Bunyamin Tar'an of the pulse research group at the CDC, we identified a population of lentil plants derived from a cross between the lentil line ILL 964a-46 and the variety CDC Robin. These parental lines carry two different sources of resistance originally derived from line ILL5588 (*Ab*R1) and the variety Indianhead (*ral*1), respectively. Line 1156-2-17A used for RNA isolation carries the IL5588 (*Ab*R1) source of resistance. Testing the markers derived from this system on a population, which contains both the sources of resistance will provide information on the utility of these markers for tracking both sources of resistance.

Table 2: List of cloned and sequenced differential display bands and their similarity to known genes in public databases

S.No	Sequence name	Band No	bp	Nucleotide sequence similarity (Blast N)	Translated protein sequence similarity (TBlastX)	Band type
1	01-1_F_(46)	1	764	No similarity	219-407 moderate similarity to *Medicago* genomic sequence	Dr
2	02-7_F_(46)	7	664	High similarity to *Medicago* pathogen induced mRNA	Very weak similarity to human and fly genomes	Ur
3	8-1	8	595	Mitochondrial transcription termination factor-like protein		Dr
4	28-2(band9)|436	9	436	240-415 strong similarity to malus cDNA and *Medicago* genomic sequences	236-430 strong similarity to malus cDNA arabidopsis cDNA and medicago genomic sequence	Dr
5	25-1(band11)|375bp	11	375	High similarity to One *Medicago sequence*	Very weak similarity to Arabidopsis PDR7 ABC transporter (96-179 and 223 to 297)	Dr
6	band12(kari3)	12	100	seq1-38-gi|16225429|gb|AF417299.1| Castanea sativa putative germin mRNA, partial cds	No similarity	Dr
7	30-2(band13)550bp	13	550	1-550 strong identity to *Medicago* sequences		Ur
8	band14	14	860	Chloroplast 50S ribosomal protein L31, putative)		Ir
9	band_15	15	895	Weak similarity to rice genomic sequences fly genome sequence (very small regions)		Dr
10	21-1	21	329	Similar to chloroplast 50S ribosomal protein L31"		Ir
11	01kari1	23	317	gi|3063395|dbj|AB012947.1| Vicia faba vcCyP mRNA, complete cds 118 1e-23		Ir
12	24-1	24	329	Chloroplast 50S ribosomal protein L31, putative)		Ur
13	31-26-1F	26	875	Weak similarity to parts of rice and fly genomes		Ur
14	28-1	28	301	gi|37994839|gb|CF806428.CF806428.1|CF806428 psHB011xl05f USDA-IFAFS:Glycine max cDNA clone sHB011l05 5, mRNA sequence: Expressed during infection of Phytophthora sojae		Dr
15	03kari3	30	315	gi|32813481|gb|AC144591.8| Medicago truncatula clone mt... 86 3e-29 2		Sr
16	03-31_F_(46)	31	646	Weak similarity to parts of to fly genome	No similarity	Dr

S.No	Sequence name	Band No	bp	Nucleotide sequence similarity (Blast N)	Translated protein sequence similarity (TBlastX)	Band type
17	33-2	33	366	gi|37962372|gb|AY261580.1| Passiflora edulis f. flavicarpa Yockteng-49 (P) glutamine synthetase GS2 (ncpGS) gene		Ur
18	34-1	34	317	gi|55274537|gb|AY618533.1| Mentha australis ribosomal protein L16 (rpl16) gene, intron		Ur
19	04kari4	37	423	gi|49170234|gb|AC146856.8| Medicago truncatula clone mt... 161 2e-37 1 ; Arabidopsis thaliana cDNA clone AD01G11 similar to (AJ012571) glutathione transferase		Sr
20	05kari5	38	423	gi|49170234|gb|AC146856.8| Medicago truncatula clone mt... 172 6e-41 1		Sr
21	06kari6	39	210	*Arabidopsis thaliana* cDNA clone AD01D01 similar to (AB005239) casein kinase I, mRNA sequence.		Sr
22	band_40	40	750	No similarity	(Large sequence region) weak (50-80% similarity to Botrytis cineraria fungal sequence at protein level: gene possibly belonging to pathogen	Dr
23	07kari7	41	349	gi|6653076|gb|AF155753.1| Pisum sativum clone Psat2-14 ... 103 3e-28 3		Sr
24	band47(kari5)	47	547	10-270; VECTOR ; 270-550: gi|8052456|emb|AJ251719.1|ZMA251719 Zea mays mRNA for transcription factor (hopi gene)	gi|8052456|emb|AJ251719.1|ZMA251719 Zea mays mRNA for transcription factor (hopi gene)	Is
25	13¥13	49	333	gi|5791502|emb|AL078459.8|HSA123M24 Human DNA sequence from... 48 0.014		CS
26	band53(kari6)	53	173	weak similarity to medicago genomic sequences (18 bp)	weak similarity to maize genomic and cDNAsequences (2-138bp)	Ir
27	02¥2	59	68	Unknown mRNA linear EST 23-MAY-2002		Sr
28	band62(kari7)	62	347	weak similarity to medicago and arabidopsis genomic sequences (17-230 bp)	strong similarity to medicago and arabidopsis genomic sequences ()	Sr
29	band66(kari8)	66	333	weak similarity to tomato cDNA (290-331) and arabidopsis genomic sequences (157-186 bp)	130-329 v strong similarity to soybean Q9SD69 BETA-D-GLUCAN EXOHYDROLASE-LIKE PROTEIN	Sr
30	14¥14|trim|195	87	179	gi|19909113|gb|AF488771.1| Bromus tectorum acetolactate syn... 167 1e-38		Ir

S.No	Sequence name	Band No	bp	Nucleotide sequence similarity (Blast N)	Translated protein sequence similarity (TBlastX)	Band type
31	17¥17	110	330	similar to chloroplast 50S ribosomal protein L31" gi|3885512|gb|AF084201.1| AF084201 Medicago sativa clone... 102 7e-20 1		Ir
32	18¥18_	112	316	No strong similarity to existing genes. Fragments similar to different regions of rice, arabidopsis and other genomes		Ur
33	19¥19	113	317	No strong similarity to existing genes. Fragments similar to different regions of rice, arabidopsis and other genomes		Ur
34	22¥22	115	347	>gi|45268048|gb|AC139 747.15| sequence spanning the HSP Medicago truncatula clone mth2-9j10, complete sequence; >gi|45434601|gb|AC13845 1.15| sequence spanning the HSP Medicago truncatula clone mth2-24k1, complete sequence ; gi|49034930|gb|AC124953. 32| Medicago truncatula clone mth2-... 54 2e-04		Sr
35	06¥6|tri m|174	130	174	gi|14625601|emb|AL389 916.15| Human DNA sequence from clone ... 287 4e-75		Cr

Discussion of results and conclusions:

Differential display methods were successfully used on RNA extracted from experimental Lentil tissue to produce 152 individual cDNA tags that represent differentially expressed genes of lentil. Seventy of the bands were re-amplified and cloned . We were successful in obtaining good sequences of 55 differential display bands. Of these, 35 sequences obtained from the clones were shown to be similar to known DNA or protein sequences of plant and/or other genomes deposited in public databases. Twenty of these differential display bands exhibited no similarity to known sequences. At least some of these sequences are likely to be similar to as yet unsequenced parts of legume genomes. One of the sequences appears to be similar to that of a fungal gene, which may indicate the feasibility of isolating virulence related genes from pathogens in infected plant tissue.

A majority of the bands isolated and sequenced by this technique are either induced or upregulated by the inoculation with the pathogen *A. lentis* isolate No.80982. Some of these genes have exhibited similarity to known genes induced by pathogens in soybean and *Medicago truncatula*. These gene sequences are candidates for expression markers

distinguishing the presence of resistance genes in lentil lines. However, the expression pattern of these genes has to be confirmed by northern blots or real time PCR before they can be used as a screening tool.

Once confirmed, PCR primers designed on the basis of these differentially these sequences can be used to test for the presence or absence of the corresponding gene transcripts in RNA isolated from inoculated plants. It is possible that this technique might detect the presence of multiple resistance genes operating through different mechanisms in the same plant. This technique is therefore potentially useful in early screening for disease resistance in genetic backgrounds containing several pyramided resistance genes. With this aim, we have identified a lentil RIL population segregating for resistance genes *ral1* and *Ab*R1, the two major gene sources for *Ascochyta* resistance used in the CDC lentil breeing program. Several RILs from this population carrying either or both resistance genes as well as exhibiting strong, moderate and poor resistance to *Ascochyta* lentis isolate 80982 were identified and tissue was collected from inoculated plants. RNA extracted from these plants can be used in future to test if the usefulness of the sequences generated by this project in differentiating between the two sources of resistance.

In addition to potential use as screening tools, the sequences may be used as a starting point for generating as sequenced tagged polymorphic sites used for mapping valuable traits in lentil. The potential for such a use of these sequences is especially significant because there are only 87 lentil sequences available in public databases as of March, 2005. It is notable that none of the sequences isolated by our project were previously reported in lentil. This project thus complements the efforts going on in other pulse research groups in generating DNA sequences of lentil, for example at University of Melbourne (EST project aimed at isolating ascochyta induced genes in lentil, Dr. Rebecca Ford's laboratory), at University of California , Davis (Choi et al. PNAS, 101, 15289-15294), and in dry bean at Agriculture and Agri-Food Canada at Harrow, ON (cDNA AFLP project to isolate genes related to bacterial blight in beans, Chaudhary et al. Poster presented at the 5th Canadian Pulse resrearch workshop, London Ontario, Nov. 28th – 30th 2004).

Papers, presentations, training and conferences:

1) Dr. Perumal Vijayan and Kari Lynn McGowan participated in poster session at Pulse Days 2005, Saskatoon, January.
2) Dr. Perumal Vijayan and Kari Lynn McGowan participated in the 5th Canadian Pulse Research Workshop, London ON, November, 2004. Dr. Perumal Vijayan made an oral presentation of the results of this project.
3) Dr. Perumal Vijayan presented the results of this project as a poster and a workshop presentation at the joint conference of International Congress on Legume Genomics and Genetics and the 5th European Conference on Grain Legumes, Dijon, France, June 2004. His workshop attendance was partly supported by travel money from this grant.

4) A poster was presented at the NAPIA meeting held at Sacramento California, 24-25 Oct. 2003 detailing the work done in this project.
5) A poster titled 'Plant genomics in lentil breeding: Development of a cDNA based rapid screening method for Ascochyta blight resistance in lentil was presented at the 4th Canadian Pulse Research Workshop held at Edmonton, Alberta, December 8-9, 2002.
6) Dr. Perumal Vijayan attended the Canadian Genetics Disease Network sponsored workshops on Bioinformatics (Proteomics) held at Windsor, Ontario, April 29 – May 4, 2002. His workshop attendance was partly supported by travel money from this grant.

ADF and SPG sponsorship of this project was duly acknowledged in all poster and oral presentations.

Indications for further development:

Based on differential display experiments, we identified and sequenced a number of gene sequences whose expression differentiates the resistant line 1156-2-17A from the susceptible variety Richlea. Primers based on these gene sequences can be developed and used in detecting the presence of resistance genes in lines carrying more than one resistance gene. In this context, the newly discovered sequences of lentil genes generated by this project can be used in screening for sequence polymorphisms in lentil varieties than can be used as markers to map various traits on the lentil genetic map.

Information of benefit to producers:

The gene sequences produced in this project can be developed for use as molecular markers for various traits in the lentil breeding program.

Personnel:

1) Ms. Satyendra Hansra: full time technician from January 23rd, 2002 - June 26th, 2002 .

2) Dr. Perumal Vijayan: Professional Research Associate from September 1st, 2002

3) Dr. Perumal Vijayan participation reduced to 50% time starting October, 2003.

4) Kari-Lynn McGowan started as technician on 50% time from October 30, 2003

Equipment – None

Fig 1. Differential display gel showing a band (No.1 arrow in lane No.9) which is expressed in control plants and suppressed in the inoculated plant (in lane No.10). The box No.2 points to a band which is induced in inoculated lentil plants (lane No. 12) and absent in the control plants (previous lane No. 11)

Lanes: 1 2 3 4 5 6 7 8 9 10 1112 13 14 15 16 17 18 19 20 21 22 23 24

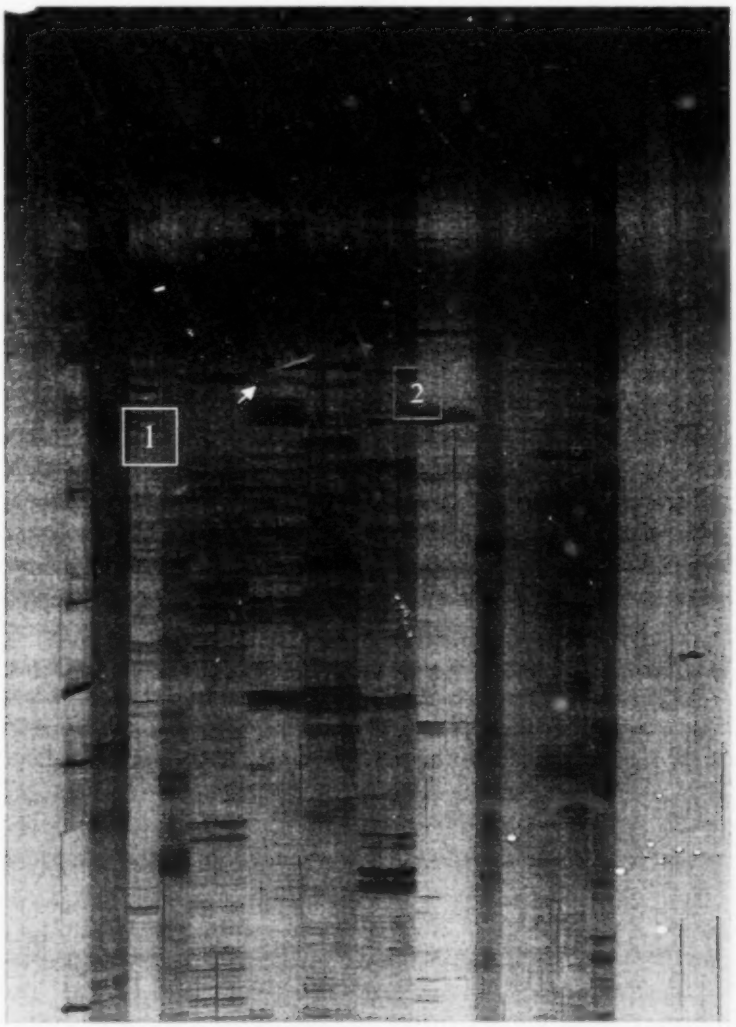
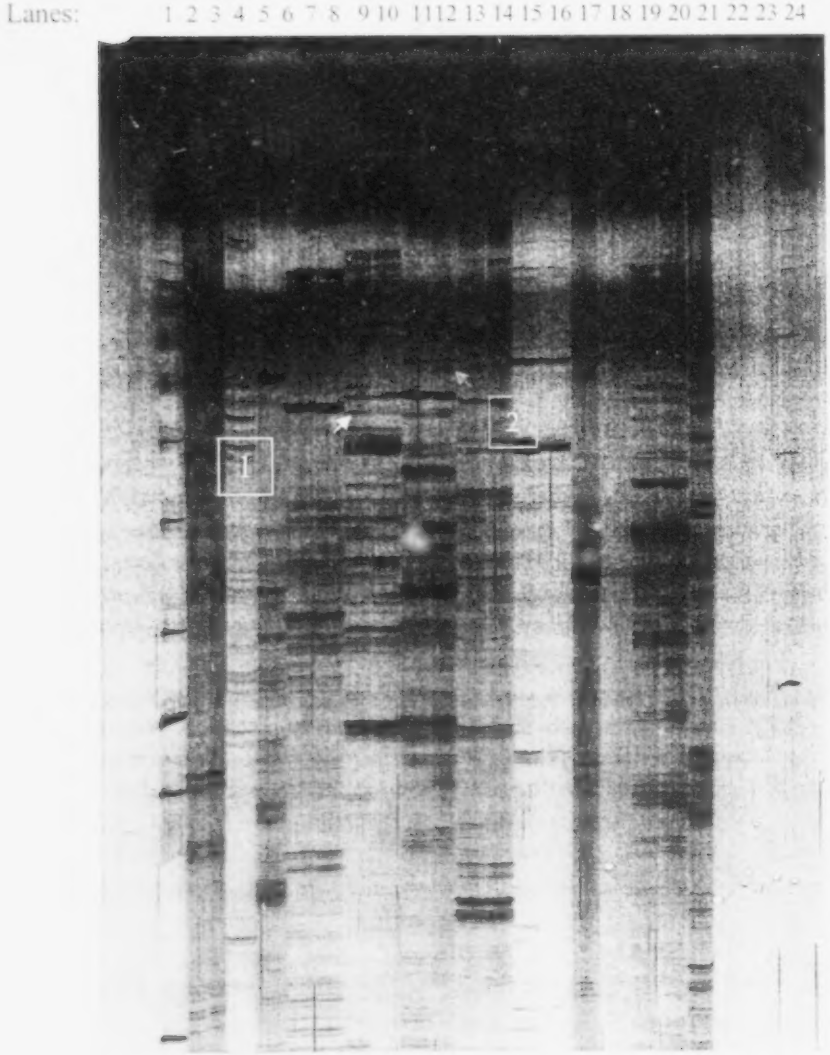


Fig 1. Differential display gel showing a band (No.1 arrow in lane No.9) which is expressed in control plants and suppressed in the inoculated plant (in lane No.10). The box No.2 points to a band which is induced in inoculated lentil plants (lane No. 12) and absent in the control plants (previous lane No. 11)



Appendix I – Examples of DNA sequences of cloned differential display bands

Sequence of Band No.7 (sequence name: 02-7_F (46) item No.2 in Table 2) is 664 base pairs long and is upregulated by Ascochyta infection in the resistant line of lentil 1156-2-17A. Basepairs 72 to 173 of this sequence exhibited strong similarity to an EST (Genbank Accession: AW776559 ; EST335624 DSIL submitted by University of Minnesota , Dept. of Plant Pathology) sequenced from cDNA library constructed from leaves of *Medicago truncatula* after inoculation with *Colletotrichum trifolii*.

Sequence of Band No.28 (Sequence Name: 28-1-3F, Item No. 14 in Table2) is 301 base pairs long and is down regulated in the resistant lentil line 1556-2-17A . Base pairs 32 to 177 of this sequence has very high similarity to a sequence (Genbank Accession No. CF807990, cDNA clone sHB031A07 submitted by Virginia Biotechnology Institute) isolated from Soybean hypocotyls infected with the pathogen *Phytophtora sojae*.

Sequence of Band No.7

>02-7_F_(46)

AAGCCTTTACGNGTGTTATTGNATTATCTAAATTTTAAATACNAGCAGAG
GCTGCNTCTGAGACNTAAATAAAAGATGAGCACAAGTGAAGAGTGGGAAT
AATTGAATTCAAATCACTGCTACTTCCTACGGNCATACGTGTCTTCAACA
CCGTCTGTAATCCTTTCTCTGCGGGCGGCAATCAANNNTAAAANNCCNCC
NTTCTTTCTTTNNCANNCTTANNTNCNTATCNCCCTTTNAANNCTTCANC
CNTTNCTNATCCCCNTNTTNNNAAAACCCCCCNNGNCNCNNTGCCGGTAN
CGCCCTTCCCCCCTCTGGNTATATTTTCCANANCCTGAAGATNCCCNTTG
GCCCCNATCCGCTNGNNAAANCTTANNNATCCCCNGCCCCCNCCCCTCCN
CCCNTTCCNTNCCTNCCGNTTNATCNTTNCCCCNTCTNNCCCNCTTCTCC
ACNNCCCTNCANCCNCCTCCTNCNANNNTTACNCACNNTTGNTCTCNNTC
CCTCCCNCTCNCCNCNNCNCCACNCNTCNTCCTTNNNGNTCCGNCNTNTC
AANANCTCCCTANNNTTCCNCCTTCCNNCCTNTCTNNNCCCTNTCCGCCG
TTCTTNCCTNCANNCTNGTCCNNNATATTTCCGTNCCTTNCTCNNCCGTN
TNNCCCNTCNNCCC

Sequence of Band No.28

>28-1-3F

ANATAAAAGATCCGAAACANCTAATTNNNGCNGTCTNGANATNTGTAAAA
GCNTCCNNANTTATGGCANANGTATCNAATNNAGTAGCTTTNNAAATAAA
AAACNGTTATNATNATNCGGANTACNNTATANCACGNGNGNGCTCCCNNC
ACTTNCGAANTCCAGGCNAGNCNANANAATTTNCTANGNTGATGGTGGTT
ANAGNTAAAANNNTTGGAAGGNTCTNTAGGGAGAAAGANANNGTTGGNGN
GANACAAANAGAGCCCANATTTNGGGGGNTANAAANATNCNANTACGNAA
CNGNAACNNATCTNTGAGCNGGNANAAGANAGC_3NNGGANCNTGNGNTN
NCGATNNTACANNTTCTNTNNTNNACATGCCAGGTGTGAGNGTACTNNGA
NNCNCTACTCNGGNGNGGGGGAACANNAANNNTGGTGNCTNTACGNGANT
NCNAANGGAATNGNCGTCCANAANANCGAGNCAGGNNNAAAATCGCGCTN
GNNTTATNTGTAANNCGACNTTTCNGGAGGANNNGNGAAANAAAAGAGAC
CGNNNGCNCCANNTTCTNTNTGNNTNTAGANGGANAAANTCGNNTNNTCA
GNACATATCNNANGGGGAAAGAGNATGNAAGAAGAGNNCNCCCTNNGATC
TCGGGNGNCNCACNCTANAAGTNGNNANNNNTNGGGANTNTGAGAGNGGG
NNAACANGAANANNCCCTNGTAACNGGGNNG